ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1985, p. 626–629
0066-4804/85/110626-04$02.00/0

Vol. 28, No. 5

Cytotoxicity of Rabbit Macrophage Peptides MCP-1 and MCP-2 for Mouse Tumor Cells

MICHAEL J.-T. SHEU, WILLIAM W. BALDWIN,* AND KENNETH W. BRUNSON†
Indiana University School of Medicine, Northwest Center for Medical Education, Gary, Indiana 46408

Received 22 May 1985/Accepted 26 August 1985

The cytotoxicity of cationic peptides MCP-1 and MCP-2 isolated from rabbit alveolar macrophages was tested against two tumor cell lines of murine lymphosarcoma origin, RAW117-P and RAW117-H10, and a normal mouse connective tissue fibroblast strain, ATCC CCL1. RAW117-H10 is a highly malignant mestastatic variant derived from the less malignant RAW117-P. Our results indicate that these peptides possess a cytotoxic activity against the tumor cells tested but not against the normal cells tested. At concentrations of 30 μg/ml, these peptides completely killed RAW117-H10 cells in suspension cultures, while inhibiting growth of RAW117-P for but a limited period of time, up to 48 h, after which growth resumed. RAW117-P cells were killed by concentrations of 40 μg/ml. These peptides showed little cytotoxicity for normal mouse connective tissue fibroblasts at concentrations of 40 μg/ml.

Rabbit alveolar macrophages, activated in vivo with complete Freund adjuvant, contain two macrophage cationic peptides (MCP), MCP-1 and MCP-2 (15). These two molecules are each single-chain polypeptides of 33 amino acids; both are rich in arginine and cystine. MCP-2 differs from MCP-1 only by substitution of leucine for arginine at residue 13 from the amino terminus (17). These macrophage peptides are bactericidal against various gram-positive and gram-negative species at near-neutral pH and relatively low ionic strength (11) and are also highly active against such fungal species as *Candida albicans* and *Candida parapsilosis* (15). They inhibit replication of herpes simplex virus and certain other viruses (R. I. Lehrer, T. Ganz, M. Sherman, and M. E. Selsted, *in* E. Pick, ed., *Lymphokines*, in press).

In these studies, we tested the cytotoxic activities of these peptides on two tumor cell lines of Abelson leukemia virus-induced murine lymphosarcoma, RAW117-P and RAW117-H10, and to one line of normal mouse connective tissue fibroblasts (ATCC CCL1). RAW117-H10 is a highly malignant and metastatic line derived from the less malignant line RAW117-P by using an in vivo sequential selection method in BALB/c mice (6). RAW117-H10 formed about 100 more liver tumor nodules per input cell than did the parent line, RAW117-P (6). This communication describes our findings of in vitro cytotoxic activity of the cationic macrophage peptides against either of the two tumor cell lines and much less cytotoxicity for the normal mouse connective tissue fibroblast strain.

MATERIALS AND METHODS

Preparation of cationic peptides. (i) Isolation and fractionation of pulmonary macrophages. Macrophages were isolated from rabbit lungs and fractionated by the procedure of Patterson-Delafield et al. (15) with some simplifications. New Zealand White rabbits (2 to 3 kg) were injected 3 to 4 weeks before collection of the macrophages with 1 ml of complete Freund adjuvant (no. 0638; Difco Laboratories, Detroit, Mich.) into a marginal ear vein. This procedure augments the number of alveolar macrophages that can be collected. Macrophages were obtained by lavaging the airways of rabbits in situ through the trachea after the animals had been sacrificed by intravenous injection of 4 to 6 ml of 3% sodium pentobarbital (Nembutal; Abbott Labortories, North Chicago, Ill.) in 10% ethanol followed by 60 ml of air. Dulbecco phosphate-buffered saline, prepared by the formula of Flow Laboratories, Inc. McLean, Va. (product catalog I, 1977) and supplemented with 5 U of heparin (Sigma Chemical Co., St. Louis, Mo.) per ml and 4.5 mM glucose, were used for the lavage, which was accomplished by serial washing with 8 to 10 50-ml volumes of buffer. These washings were pooled and centrifuged at 250 × *g* for 10 min. The sedimented macrophages were stored at −20°C.

Alveolar macrophages collected from four to six rabbits (approximately 0.5×10^9 to 1×10^9 cells) were suspended in about 5 ml of cold 0.34 M sucrose and disrupted in a Potter Elvehjem tissue grinder with a motor-driven Teflon pestle for 3 to 5 min in an ice bath. After the addition of 5 ml of 0.34 M sucrose to the homogenate, the suspension was centrifuged at 250 × *g* and 4°C for 10 min. Supernatant fluids were stored on ice, and the sediments were suspended in 5 ml of cold 0.34 M sucrose and homogenized again. All supernatant fluids were combined and centrifuged at 900 × *g* and 4°C for 10 min. The supernatant so collected was centrifuged for 20 min at 27,000 × *g* and 4°C. Sediments were stored at −20°C.

(ii) Extraction of peptides from the sediments. The sediments were extracted in a cold room four times with 5-ml volumes of 0.01 M citric acid (pH 2.7) for 2 h by the procedure of Patterson-Delafield et al. (15).

(iii) Electrophoresis. Slab gels (14 by 12 by 0.2 cm) with 15% acrylamide and a 1.5-cm stacking gel without the sample comb were prepared by using the Lehrer et al. modification (9) of the method of Reisfeld et al. (16). Between 5 and 7.5 ml of sample was loaded onto each gel and mixed with 10% (vol/vol) glycerol and 0.001% (wt/vol) methyl green. Electrophoresis was conducted at room temperature with a running water cooling system by applying a current flow of 15 mA for two gels until the dye front had migrated to the bottom of the gel (approximately 18 h). The buffer system used was 3.12% beta-alanine (wt/vol) in 0.8% acetic acid (vol/vol). After electrophoresis, 1-cm slices were cut from each vertical edge and stained with 0.2% Coomassie brilliant blue dissolved in a solution of 46.2% methanol and 7% acetic acid. Silver stain (13) was sometimes used to

* Corresponding author.
† Current address: Pfizer Central Research, Groton, CT 06340.

stain the gel. The peptides were located in two bands within 1 to 2 cm above the dye front. The gels were sliced horizontally, and the strips of gel containing these cationic peptides were pulverized and eluted twice for 16 h at room temperature with 20 ml each of distilled water. Eluates were passed through membrane filters (pore size, 0.22 nm; Millipore Corp., Bedford, Mass.) and stored at −20°C.

(iv) Protein determination. Protein content with bovine serum albumin as the standard was measured by the Bio-Rad procedure (4).

Microbiological assay of peptides. Microbicidal activity was assessed by either the colony counting method (15) or the dye exclusion method (10) with *C. albicans* ATCC 10231 as the test organism.

Effects of MCP on growth of tumor cells. (i) Cell cultures. RAW117-P and RAW117-H10 cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco modified Eagle medium (DMEM) with 10% heat-inactivated calf serum (Flow Laboratories) (7). The malignancy of the tumor cell lines was monitored by intravenous injection of 5,000 cells in 0.1 ml of serum-free DMEM into the tail vein of BALB/c mice. Animals were observed daily, and the survival rate was recorded.

(ii) Growth inhibition assay. The culture medium used in all growth inhibition assays was DMEM with 10% calf serum. All cultures were incubated as above.

One test of the cytotoxic activity, the microculture method, was performed in a 96-well tissue culture cluster plate (no. 3596; Costar, Cambridge, Mass.). The tumor cells and peptides were added to the wells so that each well contained 1,400 cells in 0.2 ml of medium; the peptide concentration was 100 μg/ml in well number 1 and serially diluted 1:2 in each well thereafter. Growth was recorded daily by microscopic observation of cell number increase (cell counts) and by monitoring the color change of cultures from red to yellow. The indicator in DMEM medium is red at pH 7.0 but becomes yellow at acidic pH. The color change of the culture from red to yellow can thus be used as an indication of cell growth. This procedure was also used to estimate approximate effective cytotoxic concentrations of peptides on tumor cells.

The growth inhibition assay was conducted in petri dishes (35 by 10 mm or 60 by 15 mm; Labware, Oxnard, Calif.) containing 2.5 or 5 ml of medium, respectively. Lyophilized peptides were dissolved in culture medium and transferred to the petri dishes. The desired number of tumor cells was then inoculated into each dish. Viable cell counts were done by diluting culture samples with 0.2% trypan blue in saline and counting the unstained cells.

(iii) Bioassay of MCP-treated RAW117-H10 tumor cells. Logarithmically growing RAW117-H10 cells at a concentration of 2×10^5 cells per ml were treated with 40 μg/ml of MCP and incubated as described above. At days 1, 2, and 5 after the treatment, cells were harvested by centrifugation, and total cell counts were performed with a hemacytometer. Five thousand cells in 0.1 ml of serum-free DMEM were injected into the tail vein of BALB/c mice. Animal survival was recorded daily.

In a second biologic assay, each mouse was injected with 5,000 MCP-treated (5 days) RAW117-H10 cells through the tail vein. Mice were killed after 2 weeks by cervical dislocation and assayed for the formation of solid tumor nodules in liver or the increase in spleen weight by the procedure of Brunson and Nicolson (6).

Effects of MCP on nonmalignant cells. Normal mouse connective tissue fibroblasts (ATCC CCL1) were purchased from the American Type Culture Collection and maintained in DMEM with 10% fetal bovine serum (7). Monolayers in cultures were trypsinized to obtain individual cells. The cell suspension was diluted at least 10-fold with growth medium and adjusted to about 5×10^4 cells per ml. A 0.5-ml sample (2.5×10^4 cells) of the cell suspension was seeded into each well of the tissue culture cluster plates (no. 3524; Costar) and incubated overnight. Culture medium was then replaced with the same growth medium plus 40 μg of MCP per ml. At regular intervals, the numbers of cells in 30 random fields in each well were counted with the aid of a Nikon inverted microscope to estimate the total cell number. To calculate the percentage of viable cells in each well, 0.3 ml of growth medium was replaced with 0.1 ml of trypan blue solution, and at least 1,000 cells were counted from each well to estimate viability.

RESULTS

Cationic peptides purified by the method of Patterson-Delafield et al. (15) gave R_f values of 0.93 for peptide MCP-1 and 0.87 for MCP-2, which are identical to those published previously (15). The peptides were extracted from the sedimented (27,000 × *g*) fraction mainly at the second and third citric acid extractions. No detectable amount of these peptides could be extracted after the fourth extraction. The prepared peptides were assayed for fungicidal activity against *C. albicans*. Whether measured by viable colony counts or the dye exclusion (10), concentrations of 40 μg/ml affected a 90% (±4%) kill in 20 min.

As measured by the microculture method (see the Materials and Methods section), growth of RAW117-P cells was not affected when cultures were treated with 25 μg or less of the peptides per ml, and RAW117-H10 growth was not affected when the protein concentration was 12.5 μg/ml or lower (data not shown).

To confirm the activity of these cationic peptides reported above, growth curve experiments were performed. For these experiments, concentrations of either 30 or 40 μg/ml were chosen. When a concentration of 30 μg/ml was used, the peptides completely killed RAW117-H10 cells in culture (Table 1) as evidenced by viable cell counts with the trypan blue exclusion method. The same concentration did not kill

TABLE 1. Effect of MCP on growth of various cells in culture

Cell line	MCP addition (μg/ml)	Viable cell count from the following no. of days after inoculation[a]			
		0	2	4	5
RAW117-P	0	3.94	5.00	6.00[b]	—[c]
	30	3.94	4.23	5.78	—
	40	3.94	4.11	0.00	—
	40[d]	3.94	3.89	0.00	—
RAW117-H10	0	3.92	4.43	—	6.08[b]
	30	3.92	3.23	—	0.00
	40	3.92	3.45	—	0.00
	40[d]	3.92	3.34	—	0.00
ATCC CCL1	0[e]	1.19	1.79	—	2.21
	40[f]	1.14	1.57	—	1.75

[a] Expressed as $\log_{10}$ (number of viable cells per milliliter), except for cell line ATCC CCL1, for which cell numbers are per microscopic field.
[b] Overgrown.
[c] —, Not done.
[d] Autoclaved MCP.
[e] 99% viability in this group.
[f] 91% viability in this group.

RAW117-P cells. The RAW117-P population, however, was completely destroyed when the concentration of the peptides was increased to 40 μg/ml (Table 1).

The heat stability of these cationic peptides was tested by autoclaving them at 121°C for 40 min and then testing them for cytotoxic activity by the above protocol. Table 1 also shows the effect of the heated MCP at a concentration of 40 μg/ml on RAW117-P cells and RAW117-H10 cells. At this concentration, the peptides killed both RAW117-P and RAW117-H10 cells and appeared to be intact after heat treatment.

Normal mouse connective tissue fibroblasts were minimally affected by 40 μg of MCP per ml with 91% viability compared with 99% viability for the untreated cells (Table 1).

To eliminate the possibility that any other constituents of the polyacrylamide gel in the area of the peptides were responsible for the cytotoxic activity, control gels were electrophoresed without protein and were eluted and assayed in exactly the same manner as above. No cytotoxic activity was observed in these control experiments.

The tumorigenicity of RAW117-H10 cells after treatment with MCP was tested by injecting treated cells into BALB/c mice (equivalent to 5,000 cells per mouse) and recording their survival time, the number of tumor nodules in the liver, and weight increase of the spleen (6). Table 2 shows the results of a host survival test used to monitor the tumorigenicity and malignancy of tumor cells. Remarkable differences in host survival rates were noted after the injection of 5,000 tumor cells into BALB/c mice. Of the mice that received RAW117-P cells, 83% were still alive 50 days after the injection, whereas animals inoculated with 5,000 cells of RAW117-H10 were all dead 16 days after injection, and all of the animals in this group died within a 2-day period. Although treatment of RAW117-H10 cells with MCP for 1 to 2 days delayed the death of the inoculated mice, only 16.7% of the mice survived for 27 days or longer. However, when RAW117-H10 cells were treated with the peptides for 5 days, all of the inoculated mice were still alive 50 days after the injection.

Results from further biologic assays (Table 3) also show that liver nodules were absent and spleen weights were normal in animals injected with RAW117-H10 receiving 5-day MCP treatment, whereas tumor nodules and spleen weight increases were seen in animals injected with non-MCP-treated tumor cells. These results indicate that 5-day treatment of RAW117-H10 with MCP eliminated the tumorigenicity of the tumor cells. This also confirms the results of the growth inhibition tests performed by viable counts with the trypan blue exclusion method (Table 1) in which cells treated with MCP for 4 to 5 days were all stained by trypan blue, whereas some cells treated for 2 days were still viable, although the growth was inhibited as compared with control groups.

TABLE 2. Survival of Female BALB/c mice after intravenous injection with RAW117 tumor cells

Cell line injected	Exposure to MCP (days)	No. of 50-day survivors/no. of mice inoculated	Survival (days) of mice that died[a]
RAW117-P	0	5/6	31
RAW117-H10	0	0/6[b]	15
	1	1/6	16
	2	1/6	17
	5	6/6	—[c]
None	0	6/6	—

[a] Median values are given.
[b] All the mice in this group died on day 15 or 16.
[c] —, Not applicable.

TABLE 3. Number of tumor nodules and spleen weights of female BALB/c mice after intravenous injection with RAW117 tumor cells

Day of sacrifice, injection[a]	Mean no. of liver nodules	Mean spleen weight in grams (range)
Day 0 (normal mice)	0	0.145 (0.117–0.197)
Day 14		
Untreated Raw117-H10 cells	65[b]	0.334 (0.148–0.429)
Treated RAW117-H10 cells	0	0.126 (0.118–0.151)
None (normal mice)	0	0.129 (0.106–0.144)

[a] Mice were injected with 5,000 RAW117-H10 cells or left uninjected (normal mice). Mice were sacrificed on day 0 or 14 as indicated. The treated RAW117-H10 tumor cells were treated with 40 μg of MCP per ml for the 5 days. Each group consisted of six 12-week-old mice.
[b] Range, 5 to 120 nodules.

DISCUSSION

In this study we found that these cationic peptides, at concentrations up to 40 μg/ml, killed RAW117 tumor cell lines completely after 5 days treatment. The cytocidal activity of the peptides was also evidenced by results of our biologic assays in which peptide-treated RAW117-H10 cells were injected into mice. Our data reveal that 5-day treatment of RAW117-H10 cells with MCP eliminates the tumorigenicity of these tumor cells.

Both results from microculture tests and growth curve experiments indicate that malignant RAW117-H10 cells are more sensitive to these peptides than the parental RAW117-P cells. This is opposite to the response of these cell lines to the antibiotic tunicamycin, a glycosylation inhibitor (8). Although distinct changes in tumor cell properties associated with enhanced metastasis are well documented (5; K. W. Brunson and G. L. Nicholson, J. Cell Biol. **79**:53, 1978), the reason for the different responses of these two tumor cell lines to the cationic peptides is not currently known. Our results also show that these peptides have relatively little cytotoxic or cytostatic effects on normal mouse connective tissue fibroblasts. Our results are similar to those of Lehrer et al. (personal communication; Lehrer et al., in press) who found that rabbit alveolar macrophages were significantly more cytotoxic toward a transformed cell line, simian virus 40-3T3, than its nontransformed counterpart.

It has been reported that macrophages from various sources can be activated for in vitro tumor cytotoxicity by a variety of in vitro or in vivo treatments. For instance, Zwilling and Campolito (20) reported that BCG-activated alveolar macrophages from hamsters were capable of destroying tumor cells. This tumor cell-killing capability can be stimulated with bacterial lipopolysaccharide (14). Weinberg et al. (19) observed a similar tumoricidal activity in peritoneal macrophages from mice. Macrophages collected from the mouse peritoneum elicited by trehalose diesters also have cytostatic activity against mastocytoma cells (12). Human alveolar macrophages are tumoricidal if induced by lipopolysaccharide (18). The precise molecular mechanisms involved in tumor cell killing by activated macrophages is

not completely clear. The secretion of a neutral serine protease (molecular weight, approximately 40,000), termed cytolytic protease, is closely correlated with macrophage killing of tumor cells (2), and the interaction of cytolytic protease and hydrogen peroxide, also produced by macrophages, is synergistic for tumor cell killing (1). Several mononuclear phagocyte products reportedly damage tumor cells (3). The cationic peptides reported add to the macrophage armamentarium for tumor cell destruction.

More studies are being undertaken in our laboratory to investigate the specificity and the in vivo effects of the cationic peptides, although these studies have been hampered by the low yield of peptides from rabbit macrophages.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Lake County (Indiana) Medical Center Development Agency.

LITERATURE CITED

1. **Adams, D. O., W. J. Johnson, E. Fiorito, and C. F. Nathan.** 1981. Hydrogen peroxide and cytolytic factor can interact synergistically in effecting cytolysis of neoplastic targets. J. Immunol. **127**:1973–1977.
2. **Adams, D. O., W. J. Johnson, and P. A. Marino.** 1982. Mechanisms of target recognition and destruction in macrophage-mediated tumor cytotoxicity. Fed. Proc. **41**:2212–2221.
3. **Adams, D. O., and C. F. Nathan.** 1983. Molecular mechanisms in tumor-cell killing by activated macrophages. Immunol. Today **4**:166–170.
4. **Bio-Rad Laboratories.** 1984. Bio-Rad protein assay. Instruction manual 84-0047-284. Bio-Rad Laboratories, Richmond, Calif.
5. **Brunson, K. W., and S. S. Joshi.** 1982. Properties of malignant lymphosarcoma variant cell lines associated with enhanced liver metastasis, p. 137–143. *In* K. Lapis, A. Jeney, and M. R. Price (ed.), Proceedings of the sixth meeting of the European Association for Cancer Research.
6. **Brunson, K. W., and G. Nicolson.** 1978. Selection and biologic properties of malignant variants of a murine lymphosarcoma. J. Natl. Cancer Inst. **61**:1499–1503.
7. **Freshney, R. I.** 1983. Cultures of animal cells: a manual of basic technique. Alan R. Liss, Inc., New York.
8. **Joshi, S. S., K. E. Tsaparikos, J. Loh, and K. W. Brunson.** 1983. Effects of the antibiotic tunicamycin on murine lymphosarcoma cells. Proc. Indiana Acad.Sci. **92**:323–331.
9. **Lehrer, R. I., L. G. Ferrari, J. Patterson-Delafield, and T. Sorrell.** 1980. Fungicidal activity of rabbit alveolar and peritoneal macrophages against *Candida albicans*. Infect. Immun. **28**:1001–1008.
10. **Lehrer, R. I., K. M. Ladra, and R. B. Hake.** 1975. Nonoxidative fungicidal mechanisms of mammalian granulocytes: determination of components with candidacidal activity in human, rabbit, and guinea pig leukocytes. Infect. Immun. **11**:1226–1234.
11. **Lehrer, R. I., M. E. Selsted, D. Szklarek, and J. Fleischmann.** 1983. Antibacterial activity of microbicidal cationic proteins 1 and 2, natural peptide antibiotics of rabbit lung macrophages. Infect. Immun. **42**:10–14.
12. **Lepoivre, M., J. P. Tenu, G. Lemaire, and J. F. Petit.** 1982. Antitumor activity and hydrogen peroxide release by macrophages elicited by trehalose diesters. J. Immunol. **129**:860–866.
13. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **15**:361–363.
14. **Panke, E. S., B. S. Zwilling, S. D. Somers, L. B. Campolito, and B. J. Packer.** 1982. The effects of lipopolysaccharide, BCG-immune T lymphocytes, and lymphokines on the generation of tumoricidal pulmonary macrophages in Syrian hamsters. Exp. Lung Res. **3**:81–90.
15. **Patterson-Delafield, J., R. J. Martinez, and R. I. Lehrer.** 1980. Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host defense mechanism. Infect. Immun. **30**:180–192.
16. **Reisfeld, R. A., U. J. Lewis, and D. W. Williams.** 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature (London) **195**:281–283.
17. **Selsted, M. E., D. M. Brown, R. J. DeLange, and R. I. Lehrer.** 1983. Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. J. Biol. Chem. **258**:14485–14489.
18. **Sone, S., S. Moriguchi, E. Shimizu, F. Ogushi, and E. Tsubura.** 1982. In vitro generation of tumoricidal properties in human alveolar macrophages following interaction with endotoxin. Cancer Res. **42**:2227–2231.
19. **Weinberg, J. B., H. A. Chapman, Jr., and J. B. Hibbs, Jr.** 1978. Characterization of the effects of endotoxin on macrophage tumor cell killing. J. Immunol. **121**:72–80.
20. **Zwilling, B. S., and L. B. Campolito.** 1977. Distinction of tumor cells by BCG-activated alveolar macrophages. J. Immunol. **119**:838–841.